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ABSTRACT

The aim of this study was to develop an enzyme linked immunosorbent (ELISA) assay to quantify spiggin in the three-spined stickleback. Spiggin is a glue protein produced in the kidney of male three-spined stickleback under the control of androgens during the breeding period. Disturbances of spiggin production in male fish and abnormal induction of spiggin in female fish are considered as valuable biomarkers of exposure to (anti-)androgenic chemicals. Polyclonal antibodies against a peptide sequence of spiggin (HRD 16) were used and the specificity of the antibodies was verified by Western blotting and direct ELISA experiments. By using HRD 16 antibodies and spiggin standard preparation, a competitive ELISA was set-up and validated. This assay appears sensitive, with a detection limit of 0.5 U/mL, and specific, as shown by the competition curves, obtained by serial dilution of male and female kidney homogenates, that were parallel to the spiggin standard curves. The ability of the spiggin ELISA to quantify spiggin induction was achieved by exposing male and female three-spined sticklebacks to 0.1 and 1 µg/L of methyltestosterone. The results show a significant dose-dependent induction of spiggin in methyltestosterone-exposed female fish compared to controls.

Keywords : three-spined stickleback, spiggin, androgens, ELISA

1. Introduction

Several man-made chemicals and natural substances present in the environment are able to disturb the normal physiology and endocrinology of organisms (Arukwe and Goksøyr, 1998). These substances, termed endocrine-disrupting chemicals (EDCs), have been defined as « exogenous substances that cause adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function » (Organization for Economic Cooperation and Development, 1997). Much research has been conducted on estrogenic endocrine disrupters and their environmental occurrence and effects on aquatic organisms are well described. However, less data are available on androgenic and anti-androgenic EDCs able to mimic or antagonise, respectively, the effects of the endogenous androgenic hormones.

Some studies have reported in vitro (anti-)androgenic activity in surface waters and sediment extracts of samples collected in rivers and estuaries (Thomas et al., 2002; Blankvoort et al., 2005; Jenkins et al., 2001; Soto et al., 2004). The application of « toxicity identification evaluation » (TIE) approach which combines bioassays and chemical analysis, allowed to identify the androgenic compounds implicated in aquatic environment contamination such as androstenedione (Jenkins et al., 2001). Moreover, several effluents of domestic and industrial water treatment plants discharging into environment, exhibited also androgenic activities linked to the occurrence of various androgenic EDCs such as testosterone, dehydrotestosterone, androsterone, trembolone (Thomas et al., 2002; Blankvoort et al., 2005). This contamination is liable for adverse effects in wildlife such as masculinisation of female common mosquito fish (Gambusia affinis) reflected by elongated anal fin rays, resembling the male gonopodium (Cody and Bortone, 1997) or female eelpouts (Zoarces viviparus) showed by a bias toward males in embryonic sex ratios of wild eelpout (Larsson et al., 2000 ; Larsson and Förlin, 2002). In ligth to the data available on occurrence of androgens in environment and their effects on aquatic species, further studies are required to bridge the gap of knowledges on the molecular, physiological, morphological and behavioural effects of androgenics EDCs.

For this purpose, the three-spined stickleback (Gasterosteus aculeatus), a small teleost fish, robust to contamination, inhabiting in both northern hemisphere seawater and freshwater, can be considered as a valuable model fish species. Indeed, during its breeding period, male stickleback exhibits androgen-dependent secondary sexual characters including development of kidney hypertrophy, nuptial coloration, territorial and nest-building behaviour. At biochemical level, the kidney hypertrophies under the control of androgens to produce an hydrophobic protein named spiggin. Spiggin is a 203-kDa cysteine-rich glycoprotein

synthesized, during the breeding period only, in the kidney of male fish and then secreted into the urinary bladder where it is assembled from three subunits prior its utilisation as a structural thread for nest building (Jakobsson et al., 1999 ; Jones et al., 2001). The spiggin gene is present, but remains silent, in female and juvenile stickleback. Following stimulation by androgens or their mimics, spiggin is readily induced. Because spiggin synthesis appears to be androgen-specific, the induction of spiggin in female or immature fish can serve as a sensitive and reliable biomarker for exposure to androgen receptor agonists in laboratory (Katsiadaki et al., 2002) and field studies (Allen et al., 2002). Spiggin can also be used as end-point for the rapid and sensitive screening of androgenic and anti-androgenic endocrine disrupting compounds tested on stickleback kidney cell culture assay (Bjorkblom et al., 2007 ; Jolly et al., 2006). Moreover, an in vivo test, based on measurement of spiggin reduction in androgen-treated female sticklebacks, has been developed for the detection of environmental anti-androgens (Katsiadaki et al., 2006).

The present paper describes the development and validation of a competitive enzyme-linked immunosorbent assay (ELISA) based on utilisation of antibodies against a synthetic peptide specific to three-spined stickleback spiggin sequence.

2. Materials and methods

2.1. Fish maintenance and spiggin induction

Three-spined sticklebacks and nine-spined sticklebacks (Pungitius pungitius) used in this study were male and female adults sampled, outside their breeding period, from uncontaminated outdoor lotic mesocosms located at INERIS (Verneuil en Halatte, France). Prior to the onset of experiments, fish were acclimated in conditions for one week. The water temperature was 15°C ± 1°C and the photoperiod was a light/dark cycle of 8/16 hours. Male and female zebrafish (Danio rerio), a fish species in which spiggin synthesis is not observed, were sampled from a laboratory stock and acclimated to a temperature of 26.5°C ± 0.4°C and a 14/10 hours light/dark photoperiod. During this period, water was completely renewed and food was supplied every other day.

Spiggin induction. Male and female fish were randomly distributed in 15 litres glass aquaria loaded to one fish per litre and exposed, during 21 days, to 20 µg/L of waterborne 17α-methyltestosterone (17α-MT) dissolved in dimethylsulfoxide (0.002 % of DMSO by aquarium). The 17α-MT concentration was chosen to allow a strong spiggin induction. The

control group received no added MT and DMSO. During this exposure, water was completely renewed and food was supplied every other day.

2.2. Standard and samples preparation

After exposure, fish were sacrificed, measured, weighed and sexed. Kidneys were dissected and weighed. Kidneys were dissolved in 100 mM Tris-HCl buffer (pH 8.5) containing 10 mM ethylenediaminetetraacetic acid (EDTA), 8 M urea, 2% (w/v) sodium dodecyl sulfate (SDS), 200 mM β-mercaptoethanol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) by heating at 100°C for two hours. Dissolved kidneys were stored at –80°C prior to spiggin quantification.

Spiggin purification by chromatographic method is difficult because it is an hydrophobic protein that reforms into glue. However, the use of induced biological material as a standard for coating and standard curve dilutions, appears as a useful principle previously (Katsiadaki et al., 2002). Hypertrophied kidneys from breeding male sticklebacks exposed to 20 µg 17α-MT /L during 21 days (see part 2.1) were pooled and dissolved (1:10 [w:v]) in denaturing buffer using the same method as with the samples. To minimise non-specific interactions between standard and antibodies, dissolved kidneys were centrifuged at 14 000 x g, 4°C for 15 min. Unfortunately, denaturing buffer components interfered with classical protein quantification assays such as 280 nm UV measurement, Bradford's assay (Bradford et al., 1976) or Lowry's assay (Lowry et al., 1951), and prevent protein quantification. Hence, to bridge this gap, the supernatant was assigned to 50 000 U/mL according the method described by Katsiadaki et al. (2002). Prior utilisation, aliquots of 20 µL were stored at –80°C.

2.3. Synthetic peptide and peptide antibody

A peptide (HRD-16) common to the spiggin subunits was synthesized by Innovagen AB (Lund, Sweden). The peptide sequence, HRDELIRDSKLHDHRC, corresponded to amino acid 161 to 176 in the three-spined stickleback spiggin protein (Jones et al., 2001) and was used to produce polyclonal antiserum (Agrisera, Vännäs Sweden). IgG (0.9 mg/mL) was purified from rabbit polyclonal antiserum against HRD-16 peptide (as described by Nilsen et al. (2004)).

2.5. Western Blot analysis

In a first time, samples were run in 10 % SDS-PAGE. While protein concentrations cannot be determined, gels were loaded with known quantities of tissue (50 µg for male kidney from three-spined stickleback and nine spined stickleback and 500 µg for all other samples). In a

second time, proteins were transferred to a nitrocellulose membrane followed by a blocking step with 5 % BSA in PBS overnight at 4°C. Membranes were then incubated for 1 h at room temperature with anti-HRD-16 IgG diluted 1:80 000 in PBS, 0.05% Tween 20, 2 % BSA (PBS-T-BSA), and then incubated for 1 h at room temperature with a HRP-conjugated goat anti-rabbit serum (Bio-Rad Laboratories) diluted 1:2 000 in PBS-T-BSA. Membranes were washed and developed using luminol/H_2O_2 as substrates (Amersham Biosciences).

2.6. Direct ELISA

Samples diluted in 0.05 M carbonate/bicarbonate buffer (pH 9.6) were coated in microtiter plates (96-well Nunc Maxisorp ; Nunc, Roskilde, Denmark) overnight at 4°C. The plates were then blocked for 1 h with 2 % BSA in PBS and then incubated 1 h, at room temperature, with primary antibody diluted in PBS, 1 % BSA. After this time, HRP-conjugated goat anti-rabbit serum diluted in PBS, 1 % was added for 1 hours at 37°C. The peroxidase activity was revealed by adding tetramethyl benzidine enzyme substrate (Interchim, France). After a 30 min incubation at 37°C, the enzyme reaction was stopped by addition of 50 µL of 1 M phosphoric acid (H_3PO_4). The absorbance was read at 450 nm using a microplate reader (Power $Wave_x$ – Bio-Tek instruments).

2.7. Stickleback spiggin competitive ELISA

This competitive ELISA for three-spined stickleback spiggin is based on a competition for the anti-HRD-16 antibodies between spiggin coated on the wells of a microtiter plate and free spiggin in the sample or standard solutions. The procedure described here has been optimized.

Coating. Microtiter plates (96-well Nunc Maxisorp ; Nunc, Roskilde, Denmark) were coated with 100 µL per well of stickleback spiggin standard at a concentration of 5 U/mL in 0.05 M carbonate/bicarbonate buffer (pH 9.6). The nonspecific binding (NSB) was determined using two wells coated with carbonate/bicarbonate buffer containing BSA only. The plates were incubated overnight at 4°C.

Standard and sample preincubation. Spiggin standard was serially diluted by a factor two in PBS, from 0.2 to 100 U/mL. Similarly, samples were serially diluted from 1: 50 to 1:50 000. Diluted solution were mixed (1:1) with primary antibody solution previously diluted to 1:10 000 to obtain a final concentration of 1:20 000. The maximal binding (B_0) was determined using a sample containing only antibody mixed with the buffer to make a final concentration identical to the antibody concentration present in all other samples. The mixed

solutions were incubated at room temperature during 30 min and subsequently incubated overnight at 4°C.

Blocking. The coated plates were washed three times with 200 µL per well of PBS-T and blocked with the same volume of PBS, 2% BSA for 1 h at room temperature.

Primary and secondary antibody incubation. The blocked plates were washed three times with PBS-T. After this washing step, 100 µL of standard/antibody or sample/antibody preincubates were introduced into the wells. The plates were sealed and incubated during 2 hours at room temperature. After this time, the plates were washed another three times with the same protocol. 100 µL of HRP-conjugated goat anti-rabbit serum were added in all wells at a dilution of 1:3 000 in PBS, 1% BSA. Plates were sealed and incubated at 37°C for 2 hours.

Development. Microtiter plates were washed five times with PBS-T and the peroxidase activity was revealed by adding 100 µL of tetramethyl benzidine enzyme substrate. The enzyme reaction was stopped after 30 min by addition of 50 µL of 1 M H_3PO_4, and the absorbance was read at 450 nm using a microplate reader.

Result calculation. The mean of the absorbance values for the NSB wells was subtracted from the values of all other wells on the same plate. The standard or sample dilution percentage binding was calculated using the following equation :

$$\frac{Bi}{Bo} = \left(\frac{\text{standard or sample absorbance}}{\text{maximal binding absorbance}} \right) \times 100$$

The B_i/B_0 standard data were fitted using least-squares method in a four-parameters logistic model and the equation was used to calculate the sample spiggin concentration.

2.8. Validation of ELISA method

Parallelism between standard and dilution curve. Kidney samples from control and MT-exposed male and female three-spined sticklebacks were serially diluted and the parallelism between the competition curves obtained with the samples and the spiggin standard curves was assessed.

Reproducibility. Precision and reproducibility of the ELISA method were determined using a one-way analysis of variance as described by Caporal-Gautier et al. (1992a;b). Briefly, five independent spiggin standard curves were made in duplicate and in four independent assays using four independent spiggin standard to provide information including reproducibility of standard preparation. Each curve was fitted with a four-parameters logistic model. Intra- and

interassay coefficients of variation (CV) were calculated using the following equations and expressed as a percentage for the spiggin concentration at 90, 80, 50, and 20% binding.

$$\text{CV intraassay} = \left(\frac{\text{intra group variance}^{0.5}}{\text{general mean}} \right).100$$

$$\text{CV interassay} = \left(\frac{\text{total variance}^{0.5}}{\text{general mean}} \right).100$$

Detection and quantification limits. The detection and quantification limits (DL and QL respectively) were determined using the standard dilution curve and were respectively represented by 90 and 80% binding (Brion et al., 2002).

Matrix effect. The interferences generated by the renal tissue on the spiggin quantification were assessed. Briefly, $\frac{Bi}{Bo}$ values for kidney homogenates from non-exposed zebrafish diluted 1:1, 1:10, 1:20, 1:25, 1:40, 1:50, and 1:100 were determined.

2.9. Usefulness of ELISA method to quantify spiggin induced by graded concentration of 17α-methyltestosterone

A total number of 120 male and female three-spined sticklebacks were randomly distributed in 8 glass aquaria of 15 litres loaded to one fish per litre, and exposed, during 21 days, to 0, 0.1 and 1 µg waterborne 17α-MT /L. The 17α-MT was dissolved in DMSO added in aquarium to 0.002 %. Two control groups were also monitored during this experiment including an absolute control that received water only and a solvent control that received DMSO in the same proportion as exposed groups. During this exposure, water was completely renewed every day and food was supplied every other day. After exposure, fish were sacrificed, measured and weighed. Kidney was dissected, weighed and stored in liquid nitrogen prior spiggin quantification. Nephro-somactic index (NSI) was calculated as (kidney weight / fish weight) x 100. Spiggin was quantified in dissolved kidneys of sticklebacks using the developed ELISA method. All samples were analysed in duplicate and the results were expressed as Unit of spiggin/g of fish and the induction factors were calculated.

2.10. Statistical analysis

Data are presented as mean ± standard deviation and all statistical analysis were performed with SPSS 14 software. Normal distribution and homoscedasticity of data were verified using respectively Kolmogorov-Smirnov and Levene tests. When data sets didn't have a normal distribution and/or homogeneity of variance, the data was log-transformed, using $F(x) = \log(1+x)$, prior to parametric analysis. A one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test was performed to test the position of significant differences between exposure conditions (α=0.05). Male and female differences were analysed using a t-test (α=0.05).

3. Results

3.1. Antibody characterisation

The ELISA method developed in this work is based on the use of antibodies against synthetic peptide from the spiggin sequence (Jones et al., 2001). Prior to ELISA development and validation, the specificity of antibodies (anti-HRD-16) were assessed. The antibodies were initially characterised by western blot analysis using induced male kidney and liver and non-induced female kidney from stickleback and also BSA and HRD-16 peptide. The results showed that anti-HRD-16 antibody binds proteins only in induced male stickleback kidney. No significant cross-reaction or non-specific binding were observed in other samples (Fig. 1A).

The antibodies were also characterised by direct ELISA analysis using HRD-16 peptide and various other biological samples with or without spiggin (Fig. 1B). The results indicated that anti-HRD-16 antibodies cross-react with HRD-16 peptide and kidney from induced male three-spined stickleback with a decrease of the signal proportional to the dilution of anti-HRD-16 antibodies in the range 1:4 000 – 1:250 000. All other samples registered an absorbance below 0.5, providing evidence for the specificity of anti-HRD-16 for spiggin.

3.2. ELISA development and validation

In a first step, several standard spiggin coating concentrations and primary antibody dilutions were used in a criss-cross serial dilution test. Three combinations characterised by an optimal absorbance (between 1.5 and 2), a low spiggin coating concentration and a high dilution rate of antibody were selected (Fig. 2). To evaluate the performance of each assay condition, standard curves were generated (data not show). After this step, one combination based on 5 U/mL coating concentration and 1:20 000 primary antibody final dilution was selected to establish and validate the competitive ELISA.

The assay condition described, consistently produced a standard curve with a working range of 0.8–4.3 U/mL (80–20% binding) in diluted samples, with 50% of binding at approximately 1.7 U/mL. The specificity of the assay was assessed by comparing the slopes of standard curves and serially diluted samples (Fig. 3). The kidney of breeding male and 17α-MT - exposed female sticklebacks showed a good parallelism with the spiggin standard within the working range of the assay. Conversely, HRD 16 peptide showed a lack of parallelism with the other samples which reflected a significant variation of cross-reaction of between antibodies and natural or synthetic ligands (data not shown). Under these conditions, intra- and inter-assay coefficients of variation were calculated for the spiggin standard curve at 90 %, 80 %, 50 % and 20 % binding. The results are presented in Table 1. All coefficients of variation were satisfactory with a value below 20 % reflecting the reproducibility and the repeatability of the method including standard preparation. The detection and quantification limits of this assay were 0.5 and 0.8 U/mL respectively.

The matrix effect linked to other fish proteins or higher concentration of denaturing buffer was assessed. Diluted kidney samples of non-exposed zebrafish containing no spiggin were used to determine $\frac{Bi}{Bo}$ values. The results presented in Table 2 show that dilutions of sample below 1:25 provide a quantifiable signal. Hence, to avoid the matrix effect, a dilution of sample of at least 1:25 was chosen for the routine assay. Consequently, the detection and quantification limits for spiggin in kidney samples were 12.5 and 20 U/mL respectively.

3.3. Spiggin induction by graded 17α-MT concentrations

In this experiment, NSI and spiggin concentration was not gender dependent parameters. However, male and female data were treated separately in light to strong physiological differences between both genders (Fig. 4). Non-exposed sticklebacks exhibited low values of NSI (0.3 ± 0.2 % for absolute control females to 0.6 ± 0.3 % for solvent control males) and no significant differences was observed between both control groups. After 21 days of exposure to 17α-MT, fish showed a significant increase of NSI values for both genders and both tested concentrations (1.1 ± 0.3 % for 0.1µg/L exposed females to 1.7 ± 0.3 % for 1.0µg/L exposed males). However, the response of NSI appeared as not dose-dependent and no significant differences was recorded between both 17α-MT exposed groups. Spiggin level in non-exposed sticklebacks was not detectable but 20 % of male and female fish exposed for 21 days to 0.002% DMSO exhibited a low level of spiggin with 24.2 ± 19.0 and 15.3 ± 7.8 U/g of fish respectively. Mean measured concentrations of spiggin in males exposed to 0.1 and 1

µg/L of 17α-MT were respectively 1303.5 ± 903.7 U/g of fish and 7645.9 ± 1111.3 U/g of fish. In female exposed to 0.1 µg/L of 17α-MT, the measured spiggin concentration was 2005.2 ± 2027.2 U/g of fish but 2 out 15 females were not induced. In female exposed to 1 µg/L of 17α-MT, spiggin was detected in all fish and the measured concentration was 5776.1 ± 2362.1 U/g of fish.

4. Discussion

Several methods have been described to assess the androgenic response of stickleback. Among them, the measurement of kidney epithelium height (KEH) in histological sections (Borg et al., 1993) and NSI determination (Sokolowska et al., 2004) provides a valuable information on androgen effects in kidney but appears to have low sensitivity. Very specific and sensitive methods to detect changes in spiggin mRNA, such as Northern blot analysis (Jones et al., 2001) and by real-time polymerase chain reaction (Geoghegan et al., 2005) have been developed. However, the response levels measured with these assays may not necessarily correlated with spiggin synthesis. ELISA have also been set up to measure spiggin level in stickleback kidney (Katsiadaki et al., 2002). This methodology appears to be very sensitive, less expensive than molecular biology assays and less time consuming than KEH measurement. Hence, we have focused on the establishment and validation of an immunoassay to detect and quantify spiggin in three-spined stickleback using antibodies raised against a synthetic peptide of the spiggin protein.

Competitive ELISA development requires the availability of a standard and an antibody specific of the protein of interest (Brion et al., 2002). Unfortunately, spiggin is a highly hydrophobic protein not easy to purify to obtain a pure and quantifiable standard, hence no spiggin purified standard and specific antibodies were commercially available. To bridge this methodological problem, Katsiadaki et al. (2002) developed an elegant strategy based on utilisation of a pool of hypertrophied kidneys as standard to obtain dilution curve, and a pool of nest material for coating the plates. This process diminishes interference between antibodies raised against spiggin from stickleback bladder, and coated material but not with other component of biological samples and could be induce overestimation of spiggin concentration. In the present work, we developed and validated an ELISA based on an alternative strategy. The selected antibodies were raised against a synthetic peptide. The chosen peptide corresponds to 161-176 amino acid sequence of the three-spined stickleback

spiggin (Jones et al., 2001). More recently, Kawahara and Nishida (2006) showed that spiggin is encoded by a multi-gene family named spg 1.1, 1.2, 1.3, 1.4, 2, 3 and 4. The chosen peptide is located in all the sequences deduced amino acid sequences from the seven spiggin cDNA except for spg 2 and 3 where the lysine in position 175 is replaced by arginine. The peptide-specific antibodies exhibited an high specificity as observed by Western Blot and direct ELISA analysis. Anti-HRD-16 antibodies showed a cross-reaction with spiggin of three-spined stickleback only. Indeed, no cross-reaction was noticed with non-induced kidneys of females and with liver of induced males, indicating that antibodies recognised a androgen-induced protein synthetised in kidney of three-spined stickleback. A lack of cross-reaction was also noticed with induced kidneys of nine-spined stickleback. As most Gasterosteidae, nine-spined sticklebacks produces spiggin used in gluing plant material to form a nest (Wootton, 1976). However, the lack of reactivity with anti-HRD-16 antibodies could be explained by the difference observed in spiggin amino acid sequence for these two fish species (i.e. 81 % similarity) and the lack of selected peptidic sequence in nine-spined stickleback (McDermott et al, unpublished data). Similarly, no cross-reaction was observed with sample from zebrafish : a fish species in which spiggin is not expressed. The high specificity of anti-HRD-16 antibodies for the spiggin of three-spined stickleback, allowed to use a preparation of hypertrophied kidneys from 17α-MT induced male three-spined stickleback as standard to obtain a steep standard curve and to coat the plates.

The spiggin standard used for this ELISA is a pool of hypertrophied kidneys from androgenised male three-spined stickleback dissolved in denaturing buffer and assigned an arbitrary value of 50 000 U/mL. A strong concentration of 17α-MT, a high affinity ligand for the androgen receptor widely used in aquaculture to produce predominately male populations (Pandian and Sheela, 1995) and able to induce spiggin (Katsiadaki et al., 2002), was used for fish androgenisation. The utilisation of biological material containing spiggin as standard has been previously described by Katsiadaki et al. (2002) and appears as a valuable method which mitigates the lack of absolute standard.

The performance of the ELISA was validated through a single-laboratory validation based on international guidelines for validation of analytical methods (Eurachem, 1998; Thompson et al., 2002). Several parameters including linear range of the standard curve (i.e. calibration), intra- and inter-assay variation (i.e. precision and repeatability respectively), detection and quantification limits for standard and samples but also matrix effect were assessed for the developed spiggin ELISA. The minimal acceptable performances for each parameter were not clearly defined for spiggin ELISA. However, validation of vitellogenin ELISA was well

described and the performances for each end-point of validation procedure were defined (Goksøyr et al., 2003). With intra- and inter-assay coefficients of variation below 20 % and a 6-fold working range between 0.8 and 4.3 U/mL, the developed ELISA in the present study appears as a valuable method to detect spiggin at 0.5 U/mL. Biological components of kidney samples and denaturing buffer used for sample preparation can interfere with the quantification of spiggin and a minimum dilution factor must be determined to avoid this problem. This matrix effect was measured by analysing homogenised kidneys from androgen-treated male zebrafish rather than kidneys from non-treated female three-spined stickleback that could contain low level of spiggin. By using kidney samples of 17α-MT treated male zebrafish, a matrix effect was observed despite the lack of spiggin in this fish species and a dilution of 1:25 was determined to avoid this matrix effect. Consequently, the detection and quantification limits for spiggin in kidney samples were respectively 12.5 and 20.0 U/mL. Under these conditions, in non-exposed females and in non reproductive males no spiggin is detected in kidney samples. In contrast, other studies using the method of Katsiadaki et al. (2002), were able to measure low levels of spiggin allowing statistical variability in female control groups (Andersson et al., 2007 ; Hahlbeck et al., 2004 ; Katsiadaki et al., 2002).

The developed and validated ELISA was used to quantify spiggin in control and 17α-MT-exposed stickleback. In the present work, non-exposed male and female sticklebacks maintained in non-reproductive status were characterised by low NSI values in accordance with the data previously reported in non-breeding sticklebacks (Sokolowska et al., 2004). In these fish, no spiggin level was detected. In male stickleback, spiggin induction is correlated closely with the increase of 11-ketotestosterone level during the breeding season (Mayer et al., 1990). Outside breeding period, levels of 11-ketotestosterone and other androgens such as testosterone, 11β-hydroxytestosterone, 11-ketoandrostenedione and 11β-hydroxyandrostenedione are low (Borg, 1994; Mayer et al., 1990) and could explain the lack of spiggin synthesis as shown in our study. Surprisingly, a low spiggin induction was detected in sticklebacks exposed for 21 days to 0.002 % of DMSO. This induction was quantified in 20 % of samples and the measured values were 24.2 ± 19.0 and 15.3 ± 7.8 U/g of fish in male and female respectively. However, no NSI increase was recorded in fish from solvent control group compared to absolute control that confirmed the high sensitivity of spiggin measurement. The review by Hutchinson et al. (2006) showed clearly that DMSO carrier has the potential of modulating the endocrine system. A recent study by Mortensen and Arukwe

(2006) suggested that DMSO may act as an estrogen receptor agonist using a salmon hepatocyte in vitro system. Whether DMSO is able to activate androgen receptor in stickleback (as well as in other fish species) needs to be investigated.

In sticklebacks exposed to 0.1 and 1 µg 17α-MT /L, the present assay allowed to quantify spiggin inductions generated by both tested concentrations. Parallel to high spiggin synthesis by kidney of both 17α-MT-exposed male and female, we recorded significant increase of NSI which is in agreement with the hypertrophy of the male kidney during the breeding season. Katsiadaki et al. (2002) reported a rough low observed effect concentration (LOEC) of 0.1 µg 17α-MT /L using spiggin induction as end-point. At this concentration, we observed a 54 and 131-fold induction in male and female sticklebacks respectively. These fold-induction rates were not different between males and females. The effect recorded was not dose-dependent in female due to the high variability linked to the lack of response observed for 2 out of 15 exposed females. Interestingly, these 2 females exhibited low NSI values. Conversely, spiggin induction recorded was dose-dependent. Our results indicated that, out of breeding period, male and female sticklebacks exhibited no spiggin basal level (except for few fish from the solvent control group) and that spiggin response was similar between gender in term of effective concentration of 17α-MT and induction levels. Consequently, both gender can be used without distinction to assess androgenic effects of chemicals if sticklebacks are collected during non reproductive period. Hence, this result allows to decrease the fish number used for laboratory and field experiments. Evidence for spiggin induction in sticklebacks exposed to 0.1 µg/L for 21 days suggested that spiggin provides a valuable end-point to assess the androgenic effects in fish.

In summary, a sensitive and robust competitive spiggin ELISA has been developed using antibodies against synthetic peptide and kidney samples from 17α-MT -induced male three-spined sticklebacks as standard. This method allows the quantification of spiggin in kidney of androgen-exposed stickleback with a high specificity. Hence, the present assay completed a set of biochemical biomarkers set-up in our laboratory and successfully applied to investigate biochemical responses of wild sticklebacks (Sanchez et al., 2007), and allows assessment of androgenic activity of chemicals in laboratory and to explore androgenic exposure in field-sampled sticklebacks.

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Fig. 1. Characterisation of HRD-16 antibodies. **A :** Western blot with HRD-16 antibodies diluted to 1 : 80 000 and induced and noninduced samples from different fish species. **B :** Direct ELISA performed with induced and noninduced samples from different fish species diluted to 1 :10 000 and various dilutions of HRD-16 antibodies. Lane 1 : HRD-16 peptide (1 µg) ; lane 2 : MT-induced male three-spined stickleback (G. aculeatus ; kidney, 50 µg) ; lane 3 : non-induced female three-spined stickleback (kidney, 500 µg) ; lane 4 : MT-induced male three-spined stickleback (liver, 500 µg) ; lane 5 : MT-induced zebrafish (D. rerio ; kidney, 500 µg) ; lane 6 : non-induced zebrafish (kidney, 500 µg) ; lane 7 : MT-induced male nine-spined stickleback (P. pungitius ; kidney, 50 µg) ; lane 8 : non-induced female nine-spined stickleback (kidney, 500 µg).

Fig. 2. Determination of optimal concentration of three-spined stickleback (G. aculeatus) spiggin and primary antibody (HRD-16) for the development of the spiggin ELISA. The arrows indicate the three combinations characterised by an optimal absorbance (between 1.5 and 2), a low spiggin coating concentration and a high dilution rate of antibody selected prior evaluating the performance of each assay condition.

Fig. 3. Cross-reaction of kidney samples from MT-induced male and female three-spined stickleback (G. aculeatus) and noninduced male, in the three-spined stickleback spiggin ELISA. Bi/B_0 = percentage binding.

Fig. 4. Spiggin levels (A) and NSI (B) in kidneys from male (black bars) and female (open bars) three-spined sticklebacks (G. aculeatus) non-exposed (absolute control and solvent control) and exposed for 21 days to two concentrations of 17α-methyltestosterone (MT) under semistatic conditions. The numbers refer to the number of fish in each treatment. Males and females were analysed separately and treatments shown with the same letter do not differ significantly from each other (α=0.05). nd was not detectable.

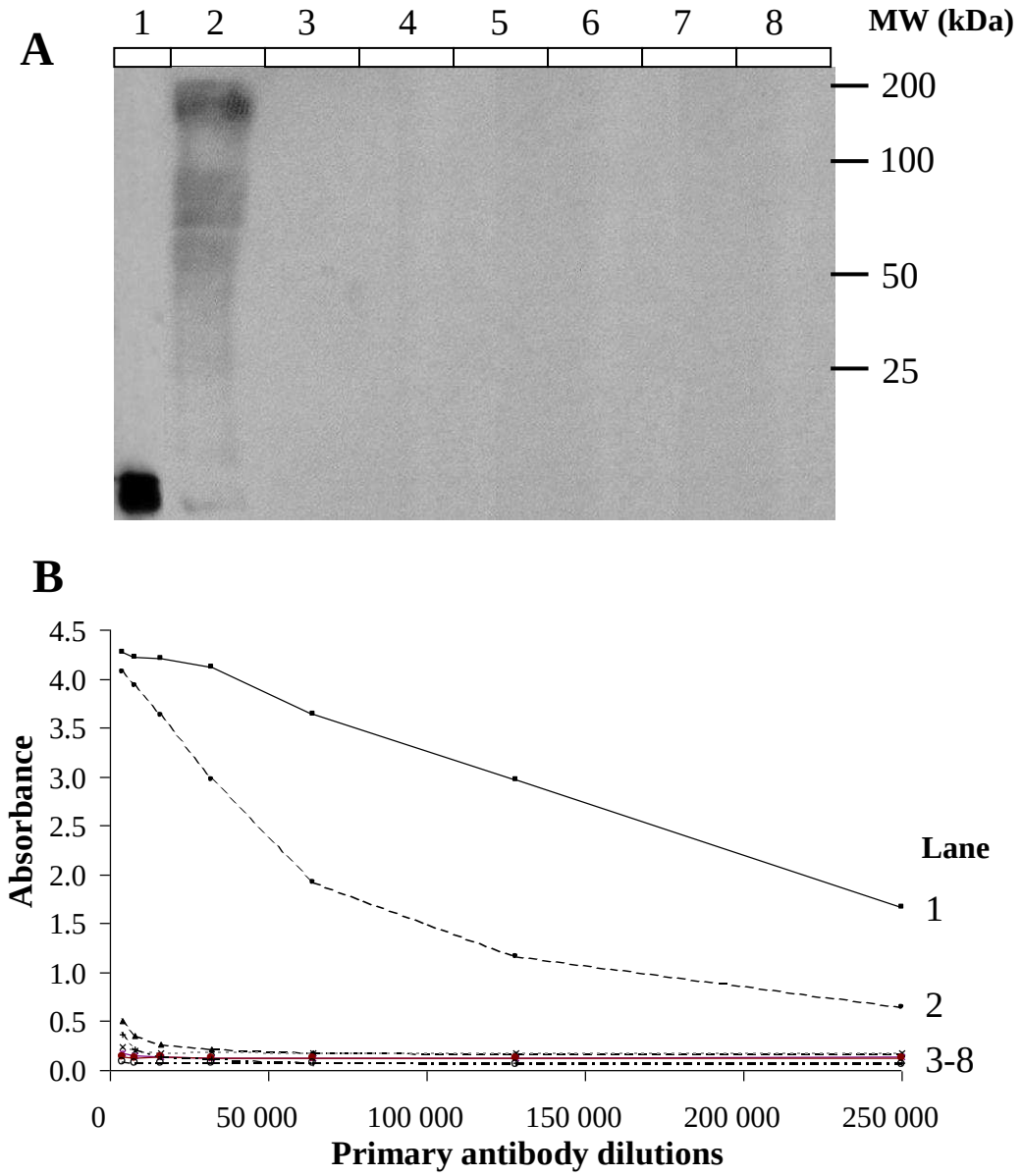
A
1
2
3
4
5
6
7
8
MW (kDa)
200
100
50
25
B
Absorbance
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
50 000
100 000
150 000
200 000
250 000
Primary antibody dilutions
Lane
1
2
3-8

Fig. 1

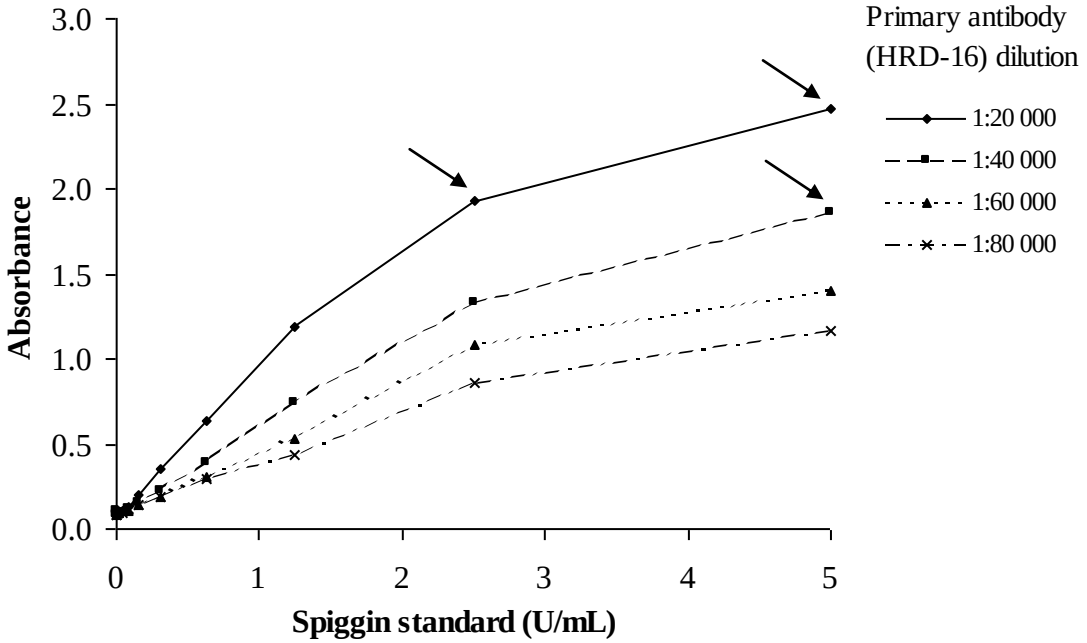
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Absorbance
0
1
2
3
4
5
Spiggin standard (U/mL)
Primary antibody
(HRD-16) dilution
1:20 000
1:40 000
1:60 000
1:80 000

Fig. 2

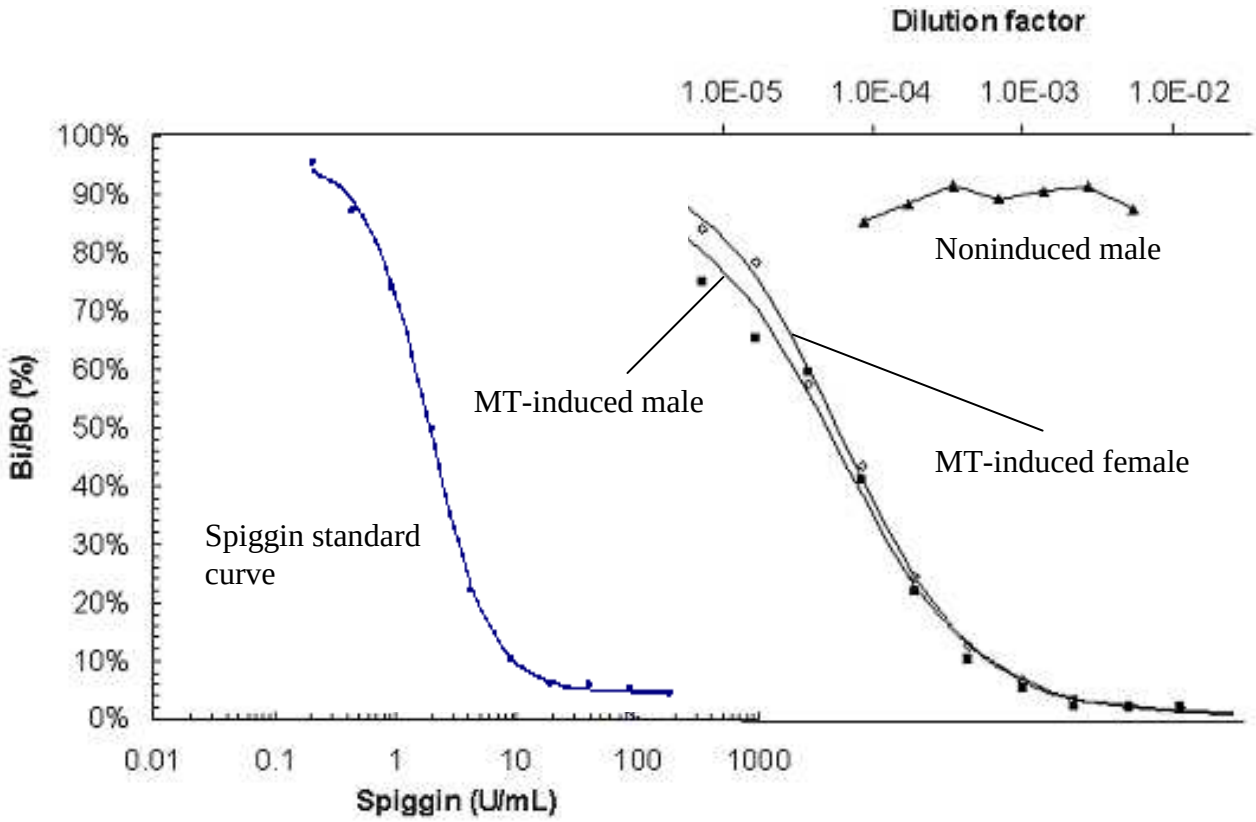
Dilution factor
1.0E-05
1.0E-04
1.0E-03
1.0E-02
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Bi/B0 (%)
0.01
0.1
1
10
100
1000
Spiggin (U/mL)
Noninduced male
MT-induced male
MT-induced female
Spiggin standard curve

Fig. 3

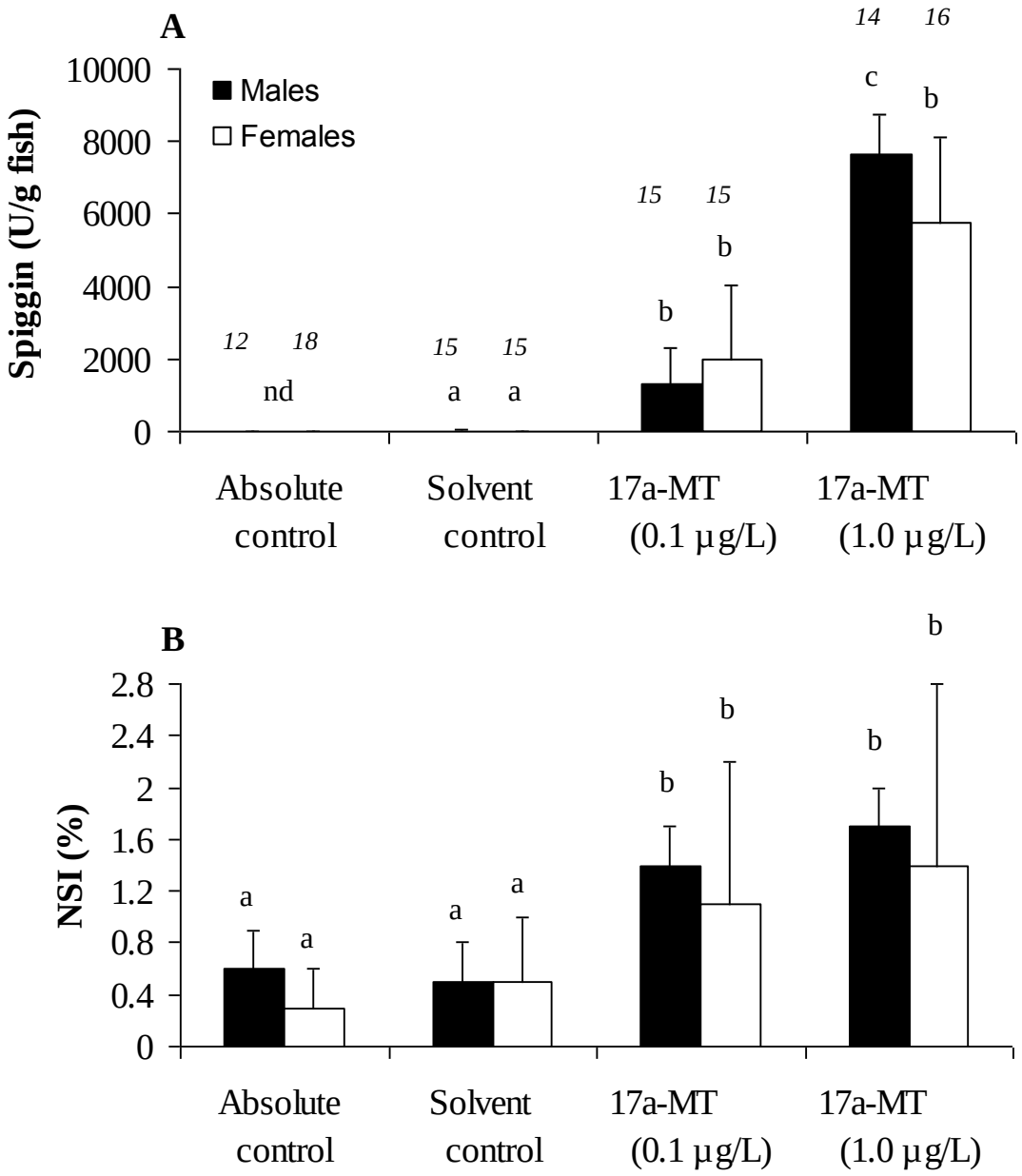
A
Spiggin (U/g fish)
10000
8000
6000
4000
2000
0
Males
Females
12
18
15
15
15
15
14
16
nd
a
a
b
b
c
b
Absolute control
Solvent control
17a-MT (0.1 µg/L)
17a-MT (1.0 µg/L)
B
NSI (%)
2.8
2.4
2
1.6
1.2
0.8
0.4
0
a
a
a
a
b
b
b
b
Absolute control
Solvent control
17a-MT (0.1 µg/L)
17a-MT (1.0 µg/L)

Fig. 4

Table 1. Characteristics of the three-spined stickleback (G aculeatus) spiggin enzyme linked immunosorbent assay (ELISA) with a combination of 5 U/mL of spiggin and antibody dilution of 1:20 000. CV = coefficient of variation.

	Binding			
	90 %	80 %	50 %	20 %
Spiggin concentration (U/mL)	0.5	0.8	1.7	4.3
CV intra-assay (%)	17.3	13.5	9.7	5.6
CV inter-assay (%)	19.2	15.1	12.2	11.2

Table 2. Determination of matrix effect using kidney sample of 17α-MT-exposed (20µg/L) male zebrafish (D rerio) diluted 1:1, 1:10, 1:20, 1:25, 1:40, 1:50 and 1:100. This tissue was chosen because it is devoid of spiggin. In the routine assay, samples were diluted 1:25 to avoid the matrix effect. Bi/B_0 = percentage binding.

Dilution of zebrafish sample	B_i/B_0 values (%)
1:1	52 %
1:10	61 %
1:20	68 %
1:25	84 %
1:40	89 %
1:50	97 %
1:100	100 %